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Reciprocal regulation of iNOS and PARP-1 during allergen-induced eosinophilia

Amarjit S. Naura, Ph.D.; Rahul Datta, M.S., Chetan P. Hans, Ph.D., Mourad Zerfaoui, Ph.D., Bashir M. Rezk, Ph.D., Youssef Errami, M.S., Mustapha Oumouna*, Ph.D., Khalid Matrougui¶, Ph.D., and A. Hamid Boulares[1], Ph.D.

Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center; ¶Department of Physiology, Tulane University Medical Center, New Orleans, LA 70112

**Present address:* Biotechnology Laboratory of Animal Reproduction, Department of Veterinary Sciences, Faculty of Agronomy and Veterinary Sciences, Blida University, BP 270 Blida 09000, Algeria.

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[1]*Corresponding Author:* A. Hamid Boulares, Ph.D.

Address: Louisiana State University Health Sciences Center, Department of Pharmacology and Experimental Therapeutics, 1901 Perdido St., New Orleans, LA 70112, U.S.A., (fax (504) 568-2361, e-mail hboulr@lsuhsc.edu).

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Abstract

iNOS inhibition was recently shown to exert no effect on allergen-challenge in human asthma, raising serious concerns about the role of the protein in the disease. **Aim**: We investigated the role of iNOS in ovalbumin-induced eosinophilia from the perspective of its relationship with poly(ADP-ribose) polymerase-1 (PARP-1) and oxidative DNA damage.

A mouse model of ovalbumin-induced eosinophilia was used to conduct the studies.

iNOS-associated protein nitration and tissue damage were partially responsible for allergen-induced eosinophilia. iNOS expression was required for oxidative DNA damage and PARP-1 activation upon allergen challenge. PARP-1 was required for iNOS expression and protein nitration, and this requirement was connected to NF-κB. PARP-1 was an important substrate for iNOS-associated byproducts after ovalbumin-challenge. PARP-1 nitration blocked its poly(ADP-ribosyl)ation activity. IL-5-reestablishment in ovalbumin-exposed PARP-1$^{-/-}$ mice reversed eosinophilia and partial mucus production without a reversal of iNOS expression, concomitant protein nitration, or associated DNA damage.

Our results demonstrate a reciprocal relationship between iNOS and PARP-1 and suggest that expression of iNOS may be dispensable for eosinophilia after IL-5 production. iNOS may be required for oxidative DNA damage and full manifestation of mucus production. Such dispensability may explain, in part, the reported ineffectiveness of iNOS inhibition in preventing allergen-induced inflammation in humans.

***Key words**:* Eosinophils; Cytokines; Allergy; Inflammation; Transgenic/Knockout Mice; Lung

Introduction

Asthma is a complex chronic inflammatory disease of the airways whose prevalence and morbidity is increasing worldwide at an alarming rate [1]. The amount of nitric oxide (NO) in exhaled air is increased in individuals with asthma and this amount reflects disease severity [2, 3]. NO is thought to play conflicting roles in airway physiology and pathophysiology [4, 5]. Indeed, NO plays a major beneficial role in airway function as it controls vascular and bronchial tone and neuroendocrine regulation of airway mediator release [3]. However, after combining with superoxide to form the highly reactive peroxynitrite ($ONOO^-$), NO rapidly oxidizes sulfhydryl groups and mediates nitration and hydroxylation of aromatic compounds including tyrosine, tryptophan, and guanosine [5]. The latter molecular changes may participate in cell demise and ultimate tissue injury. NO is synthesized by a variety of cell types, including (activated) macrophages as well as endothelial and epithelial cells. The synthesis of NO is catalyzed by each of three distinct forms of NO synthase (NOS)-neuronal NOS, endothelial NOS, and inducible NOS (iNOS, or NOS2). Whereas iNOS is either absent or present in only small amounts in most cell types and tissues under normal circumstances, a wide range of inflammatory agents including allergen exposure rapidly induce its synthesis [4, 6].

Although inhibition of NO production by iNOS appeared to be a very viable therapeutic target to prevent manifestation of asthma symptoms upon exposure to allergens [7-9], a recent clinical study by Singh's group raised serious questions on the validity of such strategy [10]. They reported that iNOS inhibition with GW274150, a selective and potent iNOS inhibitor, effectively reduces exhaled breath NO but does not affect airway inflammatory cell numbers or airway hyperreactivity after allergen challenge in subjects with asthma. Interestingly, reports by Holla et al. [11] and Batra et al. [12] show that polymorphisms in the iNOS gene may be important for asthma protection or susceptibility. Such conflicting reports undoubtedly suggest that additional studies are necessary to fully establish the intricate role(s) of NO and its metabolites during airway

inflammation. Furthermore, it is becoming clear that an examination of the role of iNOS in the context of other players of airway inflammation is necessary. This approach may not only allow for the understanding of the role of iNOS during inflammation, but also may identify alternative strategies for the treatment of asthma symptoms.

Our laboratory and others have shown the involvement of poly(ADP-ribose) polymerase-1 (PARP-1) in tissue injury and its implication in several conditions associated with oxidative stress and inflammation including allergic airway inflammation [13-16] (reviewed [17]. In addition to its effects on cell and tissue homeostasis through NAD^+ metabolism, PARP-1 is thought to participate in inflammation by regulating, directly or indirectly, the expression of several inflammatory factors including iNOS (reviewed [17-19]. Such activity has been associated with the ability of PARP-1 to regulate signal transduction events that result in the activation of NF-κB [20, 21].

In this study, we examined the role of iNOS in allergen-induced eosinophilia from the perspective of its relationship with PARP-1 and oxidative DNA damage. Our study reveals a reciprocal relationship between the two proteins. We show that PARP-1 is required for iNOS expression, and is activated by oxidative DNA damage caused by iNOS byproducts, and that PARP-1 enzymatic activity is modulated by nitration. Furthermore, our data show that iNOS may be dispensable after IL-5 production. Such dispensability may explain the reported ineffectiveness of the specific iNOS inhibitor in preventing allergen-induced inflammation in humans [10].

Methods

Animals

Mice were bred in a specific-pathogen free facility at LSUHSC, New Orleans, LA, and allowed unlimited access to sterilized chow and water. Maintenance, experimental protocols, and procedures were all approved by the LSUHSC Animal Care & Use Committee. C57BL/6 wild type and iNOS$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6 PARP-1$^{-/-}$ mice were generated by backcrossing the knockout mice under C57BL/6xSV129 mixed background with C57BL/6 wild type mice for at least seven generations. The last generation was interbred to generate the C57BL/6 PARP-1$^{-/-}$ mice.

Protocols for sensitization, challenge, organ recovery, and tissue staining

Six to eight-week old C57BL/6 wild type, iNOS$^{-/-}$ or PARP-1$^{-/-}$ mice were sensitized and challenged as previously described [22].

Animals were killed by CO_2 asphyxiation and lungs were fixed with formalin for histological analysis or subjected to bronchio-alveolar lavage (BAL). Formalin fixed lungs were sectioned and subjected to hematoxylin and eosin (H&E), Periodic Acid-Schiff (PAS) staining using standard protocols or to immunohistochemistry (IHC) with the appropriate antibodies as described previously [13].

Cell culture, immunofluorescence microscopy, immunoblot analysis, and immunoprecipitation

Naïve peritoneal macrophages were isolated using a standard protocol and cultured in RPMI supplemented with antibiotics and 10% fetal bovine serum. Cells were then seeded on chamber slides and treated with 1 μg/ml LPS (Alexis, San Diego, CA) for different time intervals. Cells were then fixed, permeabilized, and stained with antibodies to murine p65 NF-κB and with DAPI essentially as described [23]. They were then examined with a Nikon fluorescence microscope. Immunoblot analysis was conducted essentially as described [23]. Nitrocellulose filters were then

probed with antibodies to iNOS, PARP-1, or actin (Santa Cruz Biotechnologies). Immune complexes were detected with appropriate secondary antibodies and chemiluminescence reagents (Pierce, Rockford, IL, USA). Immunoprecipitation with antibodies to nitrotyrosine was conducted essentially as described [24].

Data analysis

All data are expressed as means ± SD of values from at least six mice per group unless stated otherwise. PRISM software (GraphPad, San Diego, CA) was used to analyze the differences between experimental groups by one way ANOVA followed by Dunnett's multiple comparison test.

Results

iNOS-associated protein nitration and tissue damage are partially responsible for allergen-induced eosinophilia following exposure— Although the role of iNOS in the pathogenesis of allergen-induced airway inflammation has yet to be fully elucidated [25], it is widely accepted that the potential mechanism by which iNOS contributes to disease pathogenesis is through the mediation of tissue damage as a results of the excessive production of NO and its byproduct peroxynitrite. Accordingly, we initiated our study by addressing the association between iNOS-mediated tissue damage and airway inflammation. We found that ovalbumin (OVA) sensitization and challenge were followed by airway inflammation manifested by eosinophilia (Fig. 1A-B). Such an inflammatory response coincided with robust iNOS expression and concomitant protein nitration-associated tissue damage as assessed by immunohistochemistry with antibodies to iNOS or nitrotyrosine (Fig. 1C-D). It is important to note that most of the iNOS expression detected in our model was primarily in macrophages, eosinophils, and to a lesser extent, in epithelial cells (Fig. 1C). Deletion of the iNOS gene caused a moderate but significant reduction in eosinophil recruitment to airways upon OVA challenge (Fig. 1A-B) and, as expected, no protein nitration was observed (Fig. 1D). These results suggest that iNOS and associated tissue damage (i.e. protein nitration) are necessary but insufficient for full manifestation of eosinophilia upon OVA challenge in our animal model.

iNOS expression is required for oxidative DNA damage and concomitant PARP-1 activation upon allergen exposure— To determine whether a lack of iNOS-mediated tissue damage corresponded with a lowering in tissue oxidative DNA damage, we performed immunohistochemistry on lung sections from OVA-sensitized and challenged wild-type (WT) or $iNOS^{-/-}$ mice with antibodies to 8-oxo-7,8-dihydroguanine (8-oxodG) or to the poly(ADP-ribose)

(PAR) moiety of PARP-1-modified proteins. As shown in Figure 2A, In OVA challenged mice, 8-oxodG staining was prominent in lung sections from WT mice but was almost completely absent in lung sections from iNOS-/- mice. Figure 2B shows that in OVA challenged mice, a marked PAR staining of WT lung tissue was observed which was nearly absent in iNOS-/- mice. This establishes an association between the occurrence of oxidative DNA damage and PARP-1 activation upon OVA challenge, confirming previous reports [13]. More importantly, these results suggest that iNOS may be a central participant in mediating oxidative DNA damage, ultimately by activating PARP-1 in lung tissue upon allergen exposure.

PARP-1 expression is required for iNOS expression and consequent protein nitration: a connection with NF-κB signal transduction— We then further assessed the relationship between iNOS and PARP-1 by examining the effect of a PARP-1 gene deletion on iNOS expression and the consequence of such expression in tissue injury after allergen exposure. As shown in Figure 3, a PARP-1 gene deletion, as previously reported [22], severely reduced eosinophil recruitment upon OVA challenge. It is important to note that the PARP-1 gene deletion exerted a higher impact on eosinophil recruitment compared to that exerted by deletion of the iNOS gene (compare data in Fig. 3A with that in Fig. 1B). An immunohistochemical analysis shows that while iNOS was highly expressed in lungs of OVA-challenged WT mice, its expression was severely reduced in lungs of PARP-$1^{-/-}$ mice, further supporting the notion that PARP-1 expression is important for efficient expression of iNOS (Fig. 3B). Consequently, lung sections from OVA-challenged PARP-$1^{-/-}$ mice showed practically no staining for nitrotyrosine (Fig. 3C) or 8-oxodG, indicating a complete absence of protein nitration and associated oxidative DNA damage as a result of a severe reduction in iNOS protein expression.

To determine if PARP-1 regulates the expression of iNOS through the signal transduction pathways of NF-κB in our experimental model, we examined the activation of NF-κB in lung sections derived from OVA-challenged WT and PARP-$1^{-/-}$ mice using immunohistochemistry. We show that OVA challenge induced translocation of p65 NF-κB into *the nuclei of* a number of cell types (such as macrophages) in the lungs of WT mice (Fig. 3D). In contrast, p65 translocation was not apparent in the lungs of PARP-$1^{-/-}$ mice. Translocation of p65 NF-κB was not detected in cells of tissue sections derived from experimental groups that were not exposed to OVA (Fig. 3D, left panel and data not shown).

We found that OVA challenge induced phosphorylation of I-κBα in a number of lung cells in sections derived from WT mice, with many of them having macrophage features as assessed by IHC with antibodies to the phosphorylated form of I-κBα (Fig. 3E). It is important to note that NF-κB activation has a transient nature, which justifies the lack of positive signal in all lung cells. In sharp contrast, OVA challenge of PARP-$1^{-/-}$ mice did not result in I-κBα phosphorylation in lung tissues. Taken together, these results suggest that the effect of a PARP-1 gene deletion on iNOS expression involves the signal transduction pathway of NF-κB. Using primary macrophages and lipopolysaccharide (LPS) as a stimulus, we confirmed that a PARP-1 gene deletion severely affected iNOS expression (Fig. 3F) and that such an effect was associated with a defect in NF-κB nuclear translocation (Fig. 3E).

Byproducts of iNOS enzymatic activity nitrate PARP-1 and inhibit its NAD^+-associated catalytic activity (i.e. poly(ADP-ribosyl)ation)— Protein nitration has been shown to result in inactivation of a number of enzymes and transcription factors [26]. To determine whether PARP-1 can be modified by byproducts of iNOS enzymatic activity, we subjected lung tissue extracts from control or OVA-challenged mice to immunoprecipitation with antibodies to 3-nitrotyrosine, after which

immunoprecipitates were subjected to immunoblot analysis with antibodies to mouse PARP-1. Figure 4A shows that OVA challenge did lead to nitration of PARP-1. To confirm that PARP-1 was nitrated by byproducts of iNOS enzymatic activity, we tested whether direct exposure of purified recombinant PARP-1 to $ONOO^-$ in vitro would lead to PARP-1 nitration. We show that indeed, PARP-1 was an acceptor for nitration as assessed by immunoblot analysis with antibodies to 3-nitrotyrosine (Fig. 4B), confirming our observation using the animal model. We then tested the consequence of such modification in vitro by assessing the ability of modified recombinant PARP-1 to poly(ADP-ribosyl)ate proteins derived from the liver of PARP-$1^{-/-}$ mice. Exposure of PARP-1 to $ONOO^-$ completely inactivated its enzymatic activity as evidenced by the absence of protein modification compared to samples that did not receive $ONOO^-$ (Fig. 4C). Accordingly, this potential regulation of PARP-1 by byproducts of the enzymatic activity of iNOS may represent another facet of the regulatory relationship between PARP-1 and iNOS.

Following IL-5 production, expression of iNOS is dispensable for the establishment of eosinophilia but expression may be required for oxidative DNA damage and full manifestation of inflammation-associated mucus production— We recently reported that IL-5 production may be dependent on PARP-1 expression [22]. Reestablishment of IL-5 through intranasal administration causes a reversal of eosinophilia in OVA-challenged PARP-$1^{-/-}$ mice (Fig. 5A). This model allowed us to ask whether the observed reversal in eosinophilia occurred concomitantly with iNOS expression. Reversal of eosinophilia by IL-5 replenishment in OVA-challenge PARP-$1^{-/-}$ mice was not associated with a reversal of iNOS expression (Fig. 5B) suggesting that iNOS expression may be dispensable after IL-5 production.

Oxidative DNA damage, such as the formation of 8-oxodG, is being increasingly associated with iNOS enzymatic activity in conditions other than allergen-induced inflammation [27-29]. The absence of a reversal of iNOS expression was accompanied with a complete absence of oxidative

DNA damage (Fig. 5C) and protein nitration (data not shown), which may suggest that iNOS expression may be an important participant in mediating oxidative DNA damage upon allergen exposure.

Hyperproduction of mucus by goblet cells is an important feature of asthma-associated airway inflammation that has been associated with the production of Th2 cytokines (for review [30]). Despite the fact that IL-5 replenishment reversed eosinophilia in OVA-challenged PARP-$1^{-/-}$ mice, it did not completely reverse mucus production (Fig. 5D-E). Such a partial reversal may be related, in part, to iNOS deficiency. Taken together, these results suggest that iNOS may be dispensable for eosinophilia post-IL-5 production; however, it may participate in the manifestation of tissue damage during allergic airway inflammation.

Discussion

While the function of iNOS in the process of airway inflammation has been addressed in a number of reports [31-34], its exact role still remains elusive. This is due primarily to the different roles that its product (NO) plays during inflammation. NO has been shown to function as a bronchodilator with anti-inflammatory properties, but it also has pro-inflammatory properties and can damage tissue through its ability to interact with superoxide to generate $ONOO^-$ (reviewed in [35, 36]. Clinically, however, the level of exhaled NO has been reliably associated with severity of disease symptoms in asthmatics [3]. A few reports show a direct role for iNOS in the process of eosinophil recruitment in that inhibition of iNOS, either pharmacologically or by a gene knockout, reduces airway infiltration by inflammatory cells, especially eosinophils, in models of allergen-induced lung inflammation (reviewed [25]). Further, two very recent reports showed that polymorphisms in the iNOS gene may be important for asthma protection or susceptibility [11, 12]. However, the results of a recent clinical trial conducted by Singh et al. questioned the role of iNOS during asthma and the viability of iNOS as a therapeutic target to treat the symptoms of the disease [10].

In the current study, we examined the role of iNOS in the pathogenesis of allergen-induced airway inflammation by analyzing the relationship of this enzyme with PARP-1, a DNA damage responsive enzyme and another important participant in inflammation. Our study proposes a reciprocal relationship between the two proteins which involves a requirement of PARP-1 for iNOS expression, activation of PARP-1 by oxidative DNA damages caused by iNOS byproducts, and a modulation of PARP-1 enzymatic activity by nitration. Additionally, the data suggest that iNOS may be dispensable after IL-5 production (see scheme in Fig. 6). These results may shed a *preliminary* light into the recently reported ineffectiveness of a specific iNOS inhibitor in preventing allergen-induced asthma in humans [10] given the potential production of large

quantities of Th2 cytokines such as IL-5 as a result of the repeated exposure to allergens. Accordingly, *our study provides new information on the intricate relationship between iNOS and allergen-induced inflammation and also highlights the need to think of new strategies for using iNOS as a therapeutic target for the treatment of asthma symptoms*.

Induction of iNOS *expression and the subsequent production of NO* in the lungs after allergen exposure has been of particular interest in numerous asthma-related studies [3, 37]. *It is important to note that NO can also be produced by the other two isoforms of NOS (eNOS and nNOS) [37{Dweik, 2005 #3360]*. We show that in our animal model, iNOS is expressed primarily in macrophages, eosinophils and epithelial cells as well as in smooth muscle cells (data not shown). *Additional cellular sources of NO in the lung may include endothelial cells of pulmonary arteries and veins, inhibitory non-adrenergic non-cholinergic neurones, mast cells, mesothelial cells, fibroblasts, neutrophils, and lymphocytes* [35, 37]. *It is presumed that NO produced by eNOS and nNOS may play a beneficial role while NO produced by iNOS may be detrimental [37]. This difference may be associated with the level of NO produced rather than cellular or molecular source*. Despite the clear induction of iNOS expression in the airways after allergen exposure, its role in the pathogenesis of asthma in animal models of the disease has been challenged by several studies (for a detailed review [25]). Our study provides evidence for a close relationship between PARP-1 and iNOS during airway inflammation. Indeed, maximal induction of iNOS in response to allergen exposure in vivo or to LPS treatment in vitro was highly dependent on PARP-1 expression. Reestablishment of PARP-1 expression using adenovirus-mediated gene transfer reversed iNOS expression in a cell culture system (data not shown), confirming this requirement. NF-κB signal transduction appears to be necessary for iNOS expression as is affected by a PARP-1 gene deletion. Recently, Yu et al. [38] showed in an elegant study that PARP-1 binds to the promoter of the iNOS gene and as a feedback regulation mechanism, PARP-1 is S-nitrosylated thus preventing its ability

to bind the iNOS gene promoter, leading to a down regulation of iNOS expression. However, iNOS gene down-regulation may also involve an important feedback mechanism with I-κB proteins that specifically bind NF-κB and prevent the binding of the transcription factor to the iNOS gene promoter [39]. Further, our results show that PARP-1 modification by nitration severely inhibits its enzymatic activity unraveling a new facet of regulation by iNOS byproducts on PARP-1. Evidently, such inhibition may exert numerous outcomes during inflammation. It may not only regulate expression of a number of cytokines and factors that are dependent on PARP-1, but also may modulate the response of PARP-1 to DNA damaging agents such as oxidative stress, including that mediated by $ONOO^-$. It is tempting to speculate that such PARP-1 inhibition by nitration may be considered as a feedback mechanism by which tissue and cells block continuity and persistence of inflammation.

It is noteworthy that iNOS gene deletion did not fully prevent PARP-1 activation, although it almost completely blocked the generation of 8-oxodG-related oxidative DNA damage. This result suggests that iNOS expression and the concomitant oxidative DNA damage are only partially effects of the observed PARP-1 activation after OVA challenge. The source of the additional DNA damage resulting from the remaining PARP-1 activation is currently unknown but could very well be associated with activities of additional oxidative stress-generating enzymes such as myeloperoxidases MPO or NADPH-oxidase.

Recently, Iijima et al. [32] reported an interesting connection between IL-5 and iNOS, as systemic administration of anti-IL-5 antibodies after OVA exposure was found to markedly inhibit iNOS expression and the consequent nitration of proteins in an animal model similar to ours. These findings suggest that IL-5 may induce iNOS expression. But it is not clear whether such a reduction in iNOS expression is a consequence of direct cross talk between IL-5 and iNOS or an indirect effect either through a reduction in eosinophil recruitment or by reducing the overall production of

reactive oxygen species. Our results show that administration of recombinant IL-5 was sufficient to cause eosinophil recruitment in OVA-challenged PARP-1$^{-/-}$ mice but was insufficient to drive iNOS expression. These results suggest that iNOS expression is not directly dependent on IL-5, but that iNOS participates in eosinophilia by upregulating IL-5 in our model (data not shown). Furthermore, the requirement of iNOS for the establishment of eosinophilia may be bypassed by IL-5. However, this does not preclude a role for iNOS in the damage that may be caused by iNOS expression and its byproducts after eosinophil recruitment. Indeed, our results show that iNOS may be crucial for oxidative tissue damage and mucus production to take place. Accordingly, our results provide potential additional *association between iNOS and* the process of allergic airway inflammation.

Taken together, our results support the existence of an important reciprocal *relationship* between PARP-1 and iNOS with a special connection to oxidative DNA damage and IL-5 in the process of eosinophilia during allergen-induced lung inflammation. Additional studies of these relationships may contribute to the clarification of the intricate role(s) of iNOS in asthma pathogenesis and may help in the design of new therapeutic strategies for the treatment of such condition.

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Figure legends

Figure 1. *iNOS-associated protein nitration and tissue damage are partially responsible of allergen-induced eosinophilia upon allergen exposure*. C57BL/6 wild type (WT) or C57BL/6 iNOS$^{-/-}$ mice were sensitized to OVA and then challenged with aerosolized OVA or left unsensitized and unchallenged; mice were sacrificed 48 h after challenge. Lungs were either fixed with formalin or subjected to BAL. (A) Lung sections were stained with H&E and analyzed by light microscopy; bars: 4 μm. (B) BAL fluids were collected and centrifuged; cells were then differentially stained, and eosinophils were counted. Data are expressed as total number of eosinophils per mouse; the numbers above the different bars represent percent eosinophils from total cell number collected by BAL. Data are means ± SD of values from at least six mice per group. * Difference from unchallenged mice, $p < 0.01$; # Difference from WT mice challenged with OVA, $p < 0.01$. Fixed lungs from the different experimental groups were sectioned, subjected to IHC staining with antibodies to murine iNOS (α-iNOS) (C) or to nitro-tyrosine (α-Nitro-tyrosine) (D) and observed by light microscopy. The right panel in (D) represents additional areas of lungs from OVA-treated WT mice to show nitro-tyrosine immunoreactivity in epithelial cells as well as infiltrating inflammatory cells. Bars: 4 μm.

Fig. 1

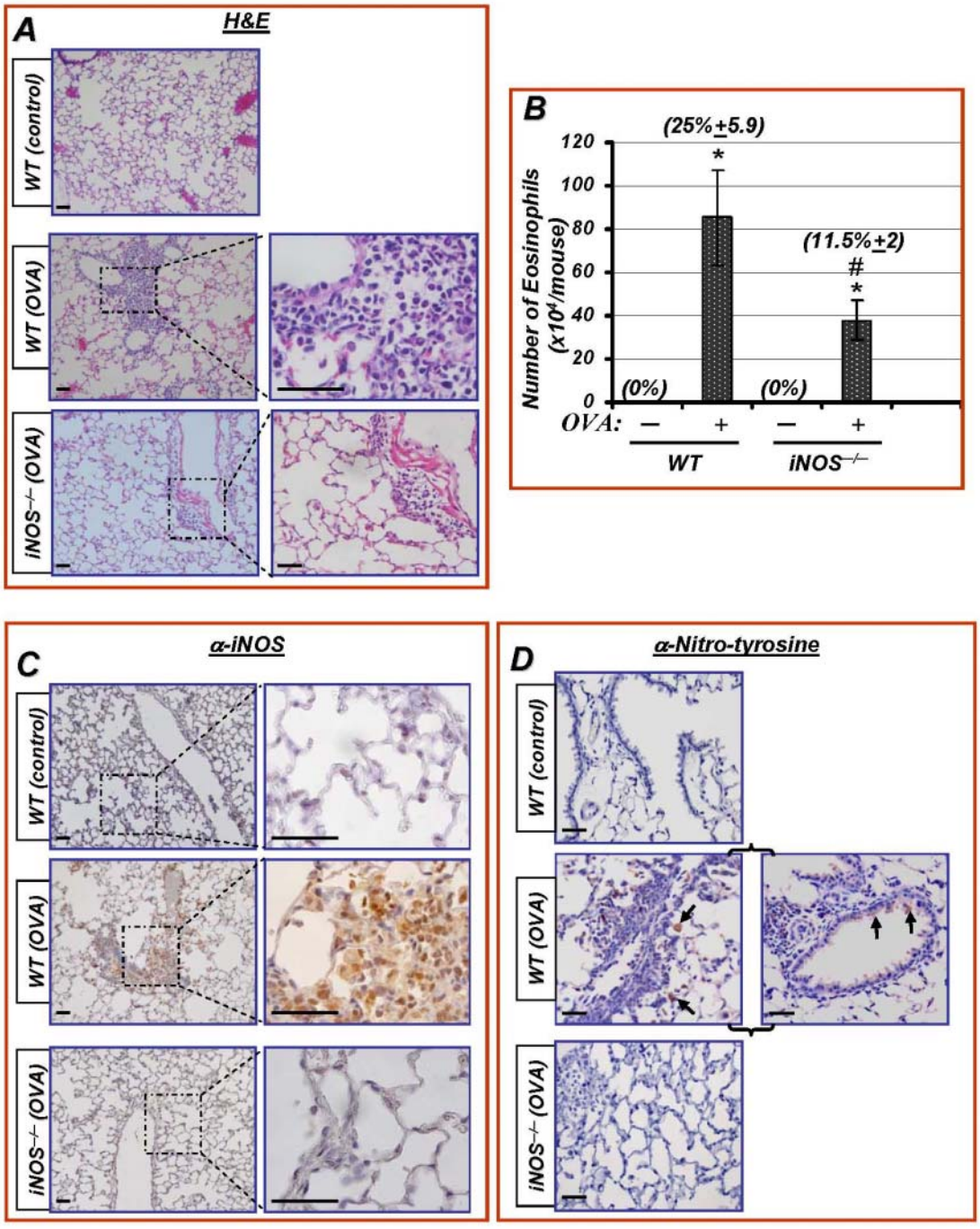

A
H&E
WT (control)
WT (OVA)
iNOS−/− (OVA)
B
(25%±5.9)
*
(11.5%±2)
#
*
Number of Eosinophils (x10⁴/mouse)
120
100
80
60
40
20
0
(0%)
(0%)
OVA: — + — +
WT
iNOS−/−
C
α-iNOS
WT (control)
WT (OVA)
iNOS−/− (OVA)
D
α-Nitro-tyrosine
WT (control)
WT (OVA)
iNOS−/− (OVA)

Figure 2. *iNOS expression is required for oxidative DNA damage and concomitant PARP-1 activation upon allergen exposure.*. C57BL/6 WT and iNOS$^{-/-}$ mice were subjected to OVA sensitization and challenge or left unsensitized and unchallenged as described for Fig. 1. Fixed lungs from the different experimental groups were sectioned, subjected to IHC staining with antibodies to 8-oxo-dG (α-8-oxo-dG) (A) or to poly(ADP-ribose) moieties of PARP-1-modified proteins (α-PAR) (B) and observed by light microscopy. Lungs sections from OVA-sensitized and challenged PARP-1$^{-/-}$ mice were used as negative controls for poly(ADP-ribose) immunoreactivity. Bar: 4 μm.

Fig. 2

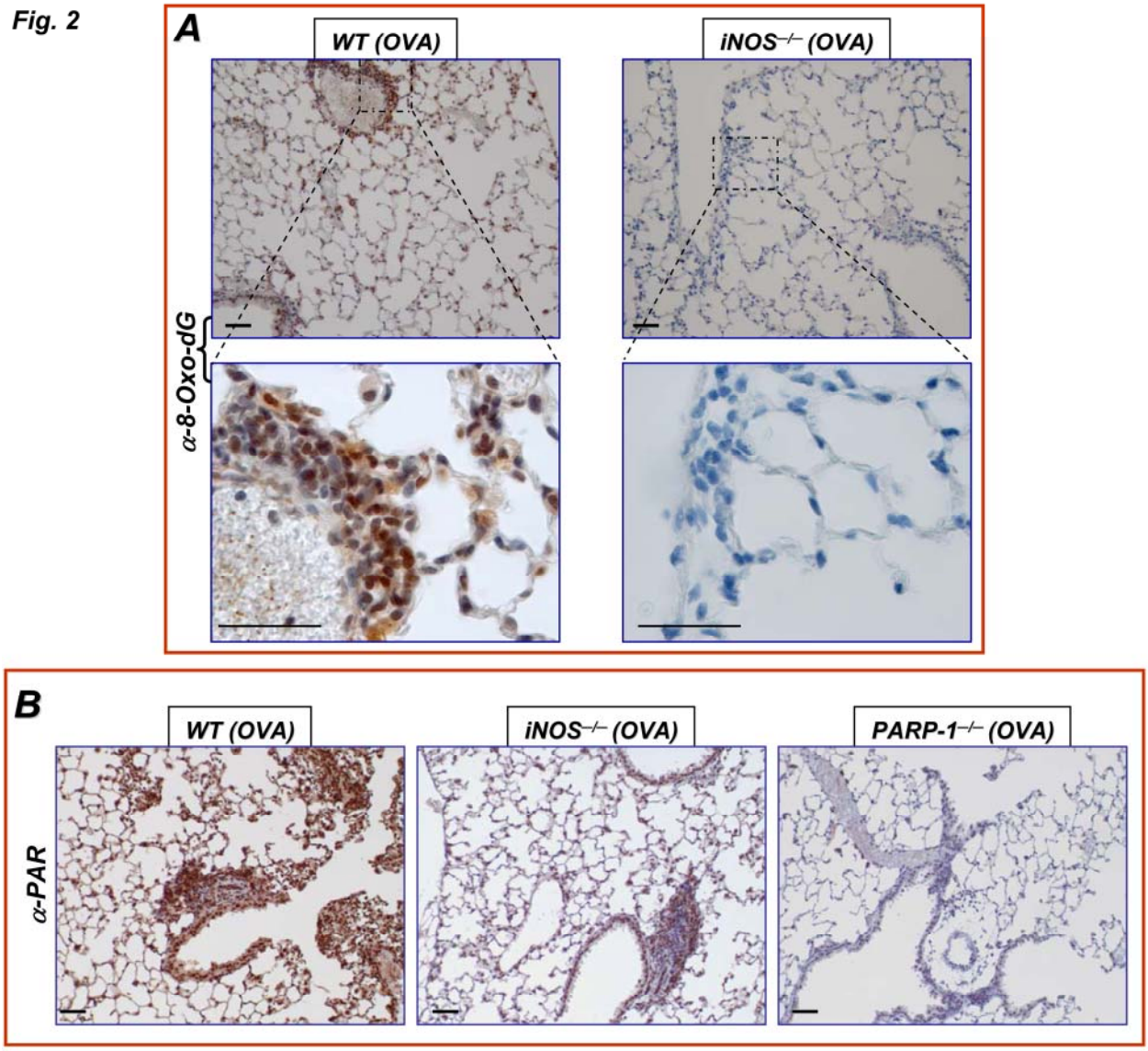


Figure 3. *PARP-1 expression is required for iNOS expression and consequent protein nitration: a connection with NF-κB signal transduction.* WT or PARP-$1^{-/-}$ mice were OVA-sensitized and challenged or left untreated. Mice were sacrificed 48 h after challenge and lungs were either subjected to BAL or fixed with formalin for IHC staining. (A) BAL fluids were collected and centrifuged; cells were then differentially stained, and eosinophils were counted. Data are expressed as total number of eosinophils per mouse; the numbers above the different bars represent percent eosinophils from total cell number collected by BAL. Data are means ± SD of values from

at least six mice per group. * Difference from unchallenged mice, $p < 0.01$; # Difference from WT mice challenged with OVA, $p < 0.01$. Fixed lungs from the different experimental groups were sectioned, subjected to IHC staining with antibodies to murine iNOS (α-iNOS) (B), to nitro-tyrosine (α-Nitro-tyrosine), or to 8-oxo-dG (α-8-oxo-dG) and observed by light microscopy. IHC analysis of lung section from control mice is not shown. Bars: 4 μm; MQ: macrophages; EC: endothelial cells. Fixed lungs from unchallenged WT mice (control), OVA-sensitized and challenged WT mice, or OVA-sensitized and challenged PARP-$1^{-/-}$ mice were sectioned, subjected to IHC staining with antibodies to murine p65 NF-κB (D) or phospho-I-κBα (E), and observed by light microscopy. Bars: 4 μm. The untreated controls which exhibited no phosphor-I-κBα immunoreactivity are not shown. (F) Macrophages isolated from peritoneal cavities of WT or PARP-$1^{-/-}$ mice were treated with 1 μg/ml LPS for 12 h, after which protein extracts were prepared and subjected to immunoblot analysis with antibodies to murine iNOS or actin. (G) WT or PARP-$1^{-/-}$ macrophages were treated with 1 μg/ml LPS for the indicated times or left untreated (control) and were then subjected to immunofluorescence staining with antibodies to murine p65 NF-κB and DAPI. Arrows indicate NF-κB nuclear translocation; magnification, x 1000.

Fig. 3

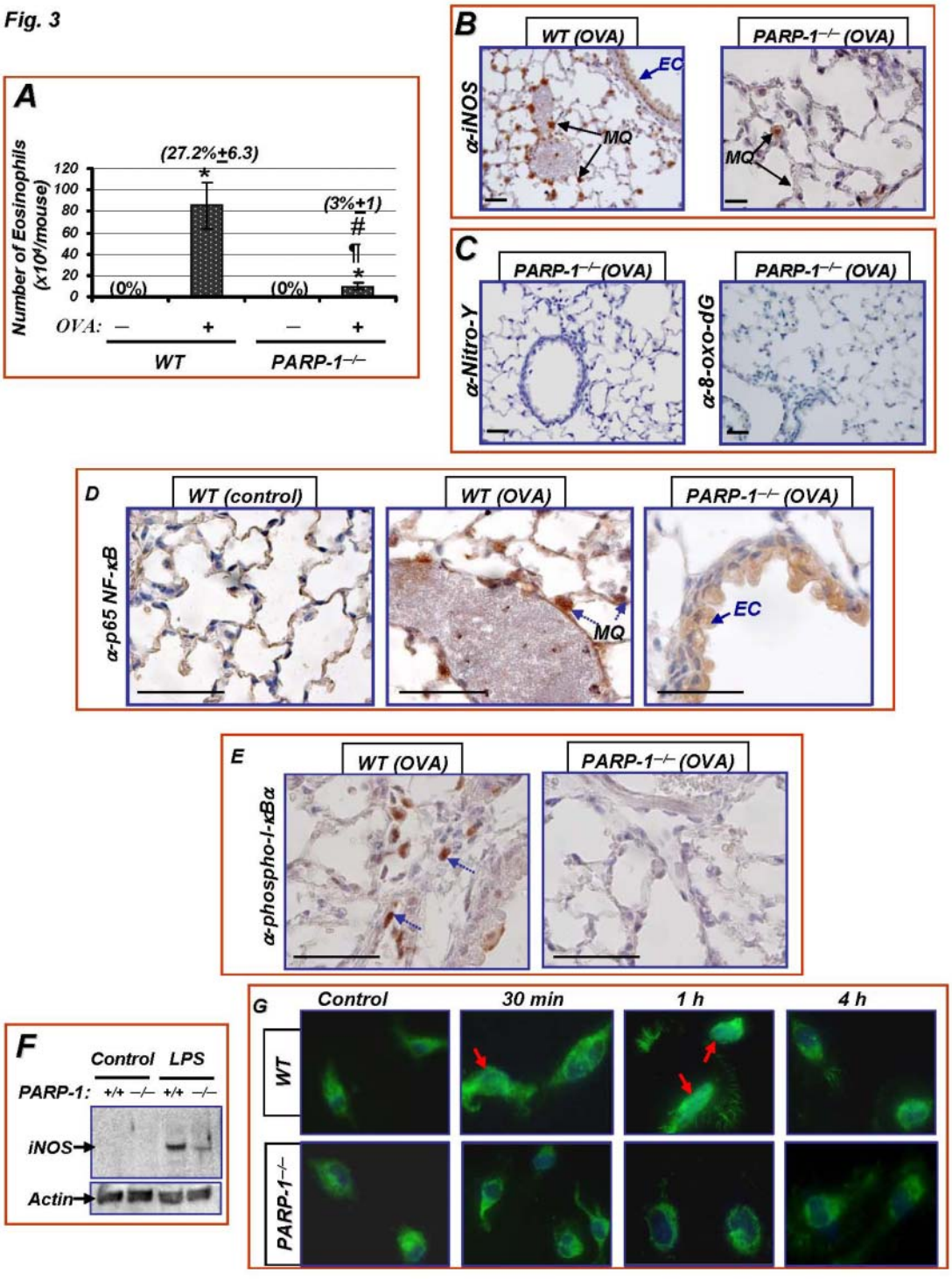

A
Number of Eosinophils (x10⁴/mouse)
(27.2%±6.3)
(3%±1)
(0%)
(0%)
OVA: – + – +
WT
PARP-1–/–
B
WT (OVA)
PARP-1–/– (OVA)
α-iNOS
EC
MQ
C
PARP-1–/– (OVA)
α-Nitro-Y
α-8-oxo-dG
D
WT (control)
WT (OVA)
PARP-1–/– (OVA)
α-p65 NF-κB
E
α-phospho-I-κBα
F
Control
LPS
PARP-1: +/+ –/– +/+ –/–
iNOS
Actin
G
Control
30 min
1 h
4 h
WT
PARP-1–/–

Figure 4. *Byproducts of iNOS enzymatic activity nitrate PARP-1 and inhibits its enzymatic activity (i.e. poly(ADP-ribosyl)ation)*. (A) Lung extracts (150 μg) from untreated or WT mice that were subjected to OVA-sensitization and challenged and sacrificed 24 h after challenge were subjected to immunoprecipitation (IP) with antibodies to nitrotyrosine (Nitro-Y) after which the precipitates were subjected to immunoblot analysis with antibodies to PARP-1. The lower panel in (A) represents a lower exposure of the blot to show the clear difference between the two samples. A 10% sample input was subjected to immunoblot with antibodies to actin to demonstrate equal loading (right panel). (B) Recombinant human PARP-1 was incubated in a nitration reaction mixture containing 10 μM $ONOO^-$ for 10 min after which the samples were then subjected to immunoblot analysis with antibodies to Nitro-Y. (C) Recombinant PARP-1 treated with 10 μM $ONOO^-$ or left untreated as in (B), in duplicates, was incubated with equal amounts of liver protein extracts derived from PARP-$1^{-/-}$ mice (see Ponceau stain) and subjected to an in vitro poly(ADP-ribosyl)ation reaction with NAD and activated (sonicated) DNA for 5 min. The reactions were terminated with sample buffer and subjected to immunoblot analysis with antibodies to poly(ADP-ribose) (PAR).

Fig. 4

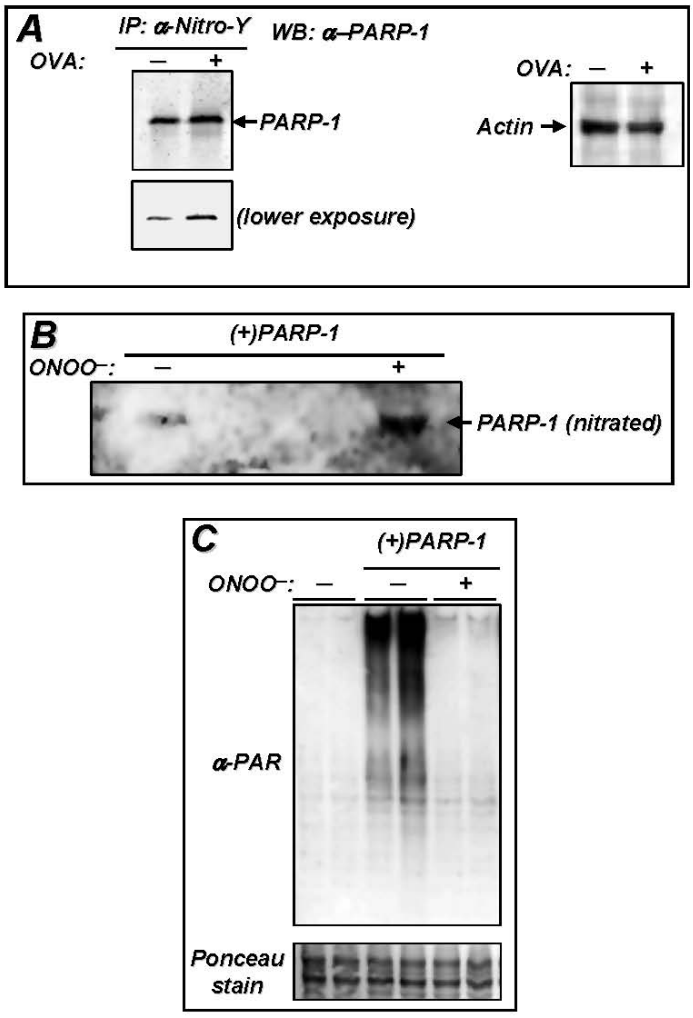

A
IP: α-Nitro-Y
WB: α-PARP-1
OVA:
–
+
PARP-1
(lower exposure)
OVA: – +
Actin
B
(+)PARP-1
ONOO⁻: – +
PARP-1 (nitrated)
C
(+)PARP-1
ONOO⁻: – – +
α-PAR
Ponceau stain

Figure 5. *Expression of iNOS is dispensable after IL-5 production for the establishment of eosinophilia and associated oxidative DNA damage but is required for full manifestation of inflammation-associated mucus production.* PARP-$1^{-/-}$ mice were sensitized to and challenged with OVA. Twenty-four hours later, mice were subjected to intranasal administration of recombinant mouse IL-5 (0.5 μg), saline, or BSA. Mice were sacrificed 48 h after the OVA challenge. (A) Fixed lungs from the different experimental groups were sectioned and subjected to H&E; arrows indicate eosinophil infiltration. Bars: 4 μm. (B) Sections from OVA-challenged PARP-$1^{-/-}$ mice that were subjected to intranasal administration of recombinant IL-5 were subjected to IHC staining with antibodies to murine iNOS; sections from OVA-challenged WT mice were used as positive controls for iNOS expression. Bars: 4 μm. (C) Lung sections from OVA-challenged PARP-$1^{-/-}$ mice that received an intranasal administration of recombinant IL-5 were subjected to IHC staining with antibodies to 8-oxo-dG; the right panel represents a higher magnification. (D) Lung sections from the latter group were subjected to PAS staining; sections from OVA-challenged WT mice were used as positive controls for mucus production. Arrows indicate sites of inflammatory cell infiltration or PAS positive goblet cells. Bars: 4 μm. (E) The extent of mucus production (hitological mucin index) was assessed using Image-Pro Plus software. *, difference from unchallenged mice, $p < 0.01$; #, difference from OVA-challenged wild type (WT) mice, $p < 0.01$.

Fig. 5

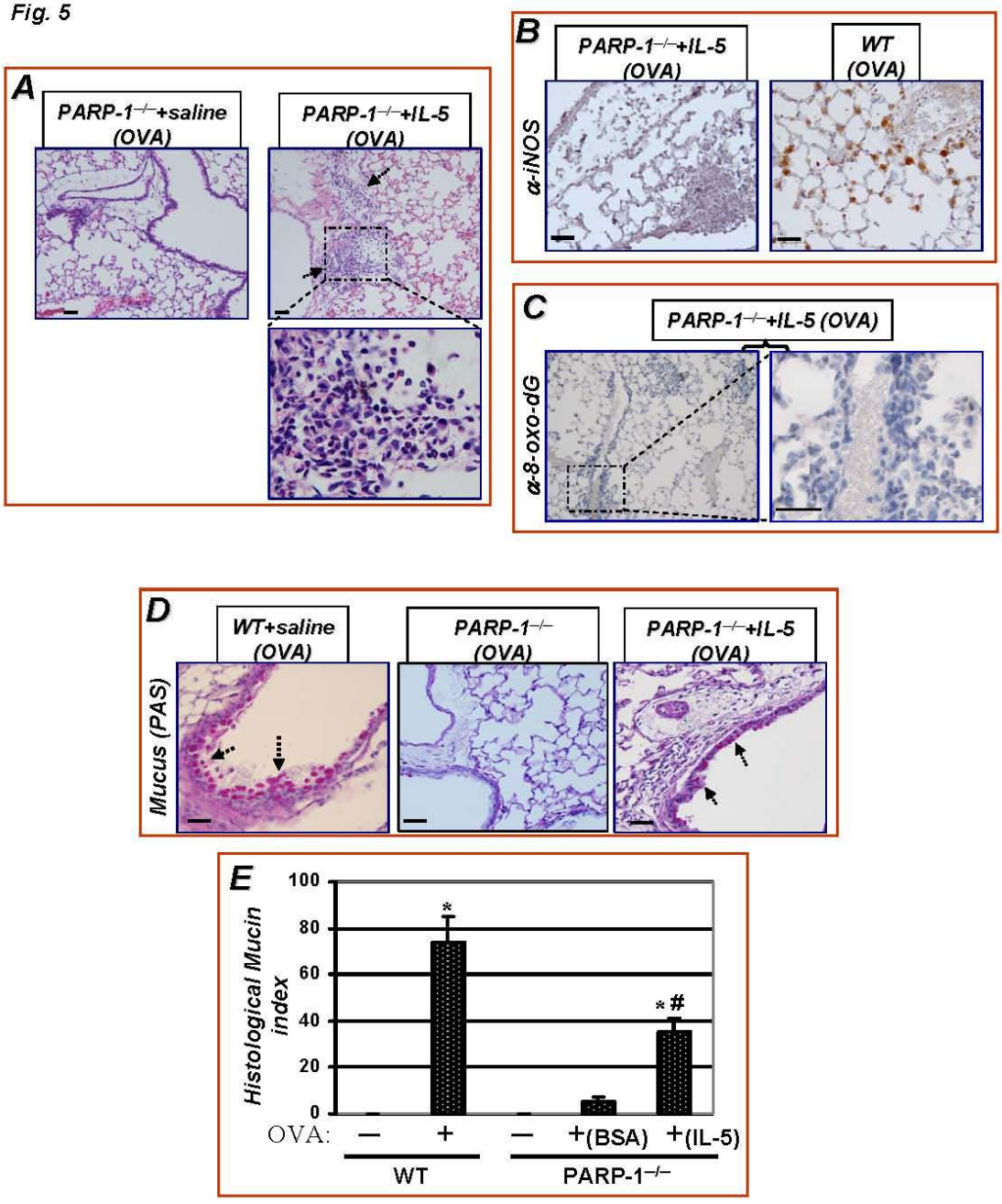
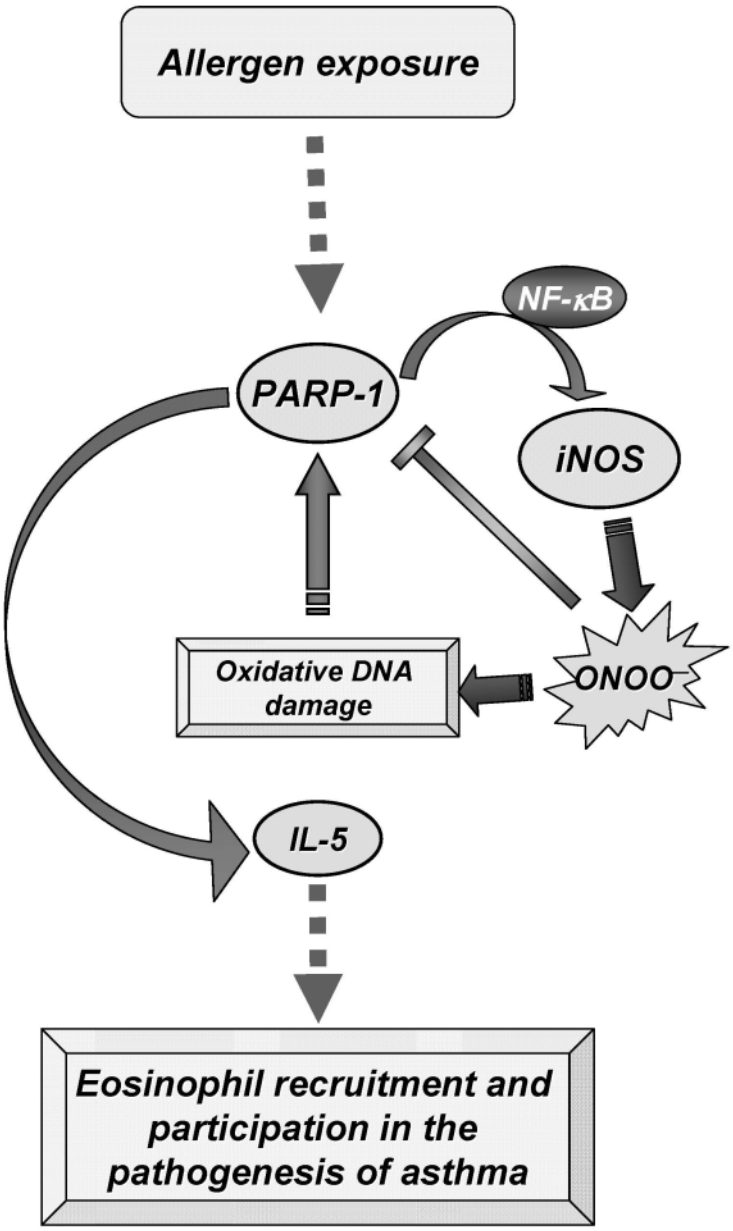

A
PARP-1−/−+saline (OVA)
PARP-1−/−+IL-5 (OVA)
B
PARP-1−/−+IL-5 (OVA)
WT (OVA)
α-iNOS
C
PARP-1−/−+IL-5 (OVA)
α-8-oxo-dG
D
WT+saline (OVA)
PARP-1−/− (OVA)
PARP-1−/−+IL-5 (OVA)
Mucus (PAS)
E
Histological Mucin index
100
80
60
40
20
0
*
*#
OVA: — + — +(BSA) +(IL-5)
WT
PARP-1−/−

Figure 6. *Model for the potential reciprocal regulation of iNOS and PARP-1 during allergen-induced airway inflammation.* Upon allergen exposure that involves a number of intricate processes, PARP-1 participates in the process of iNOS expression potentially through NF-κB-mediated signal transduction and IL-5 production through a yet unknown mechanism. IL-5 in addition to several important cytokines promotes the recruitment of inflammatory cells such as eosinophils to the lung. iNOS produces high levels of NO, which can be converted into $ONOO^-$ after its interaction with superoxide. $ONOO^-$ causes oxidative tissue damage as manifested by protein nitration and induces DNA strand breaks which are potent activators of the NAD-utilizing enzymatic activity of PARP-1. Nitration of PARP-1 renders the enzyme inactive representing a potential regulatory mechanism by which iNOS modulates PARP-1 enzymatic activity. Inhibition of PARP-1 may represent an attempt by the cells to control the inflammatory response.

Fig. 6


Allergen exposure
NF-κB
PARP-1
iNOS
Oxidative DNA damage
ONOO
IL-5
Eosinophil recruitment and participation in the pathogenesis of asthma